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13. ABSTRACT (Maximum 200 Words)

The AP-1 transcription factor is a central component of many signal transduction pathways. We have shown that blocking AP-1 by over-expressing a dominant negative form of cJun (cJun-DN, Tam67) inhibits breast cancer cell growth. We hypothesize that inhibition of AP-1 blocks the cell cycle, suppression of AP-1 in vivo inhibits the development of breast tumors, the cJun-DN protein binds and inactivates important growth regulatory proteins present in breast cancer cells, these proteins include Jun and Fos family members as well as coactivators that bind cJun. In the present study, we demonstrated that TAM67 inhibits breast cancer growth both *in vitro* and *in vivo*. We determined the mechanism by which AP-1 blockade inhibits breast cancer growth. Our studies suggested that TAM67 inhibits breast cancer growth predominantly by inducing CDK inhibitors (such as P27), suppressing G_1 cyclins expression and reducing CDKs activity, thus inducing a cell cycle block. We also showed that TAM67 binds all Jun and Fos family members, and it inhibits breast cancer cell also by interacting with cFos and preventing the recruitment of co-activators.

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Breast cancer; AP-1 transcription factor; dominant negative mutants; coactivators; cell cycle regulation; mouse xenograft study

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INTRODUCTION

The AP-1 transcription factor is a central component of signal transduction pathways in many cells. The AP-1 family of transcription factors consists of Jun (cJun, JunB, JunD), Fos (cFos, FosB, Fra1, Fra2) proteins, Jun dimerization partners (JDP1 and JDP2), and also ATF (activating transcription factor) or CREB (CRE–binding) protein families, Maf proteins, and the neural retina specific gene product Nrl (1-5). These proteins contain a leucine zipper and form either homodimers or heterodimers through this domain. The Jun family members can homodimerize or heterodimerize with the different Fos, ATF/CREB family members, as well as v-Maf and c-Maf proteins. The Fos members can only heterodimerize with the different Jun family members. Jun-Jun and Jun-Fos dimers preferentially bind to the phorbol 12-O-tetradecanoate-13-acetate (TPA)- responsive element (TRE), whereas Jun-ATF dimers and ATF homodimers prefer to bind to the cAMP-responsive element (CRE) (6). It was shown that AP-1 is involved in controlling cellular proliferation, differentiation, apoptosis, and oncogene-induced transformation. However, most of this work has been done in fibroblasts. Relatively few studies of the function of AP-1 have been performed in epithelial cells. Thus the exact role of this transcription factor family in controlling the proliferation and transformation of epithelial cells is not known. We and others have demonstrated that Jun and Fos family members are variably expressed in human breast tumors, and AP-1 is activated by a variety of important growth factors such as EGF, IGFs, and estrogen. Recent studies found correlations between high phospho-cJun expression and decreased overall survival in breast cancer (7); and the expression of FosB correlated with ER-positivity while expression of Fra1 showed a strong negative correlation with ER positivity (8). AP-1 complexes may be involved in regulating transcription of the ER gene as well (9). These results indicated that AP-1 proteins might play a role in the pathogenesis and growth of breast tumors. In addition, AP-1 activity has been shown to increase when human breast cancers become resistant to tamoxifen (10,11). The cJun overexpresson in MCF 7 breast cancer cells produces a tumorigenic invasive and hormone resistant phenotype (12,13). All of these studies suggest that AP-1 transcription factors may also be critical for the growth of tamoxifen-resistant or drug-resistant breast cancer cells. To investigate the role of AP-1 in regulating breast cell growth, we have developed MCF-7 breast cancer cell lines that express an inducible cJun dominant-negative mutant (cJun-DN) under the control of the Tet-off system. This cJun dominant-negative mutant lacks the transactivation domain of cJun, yet retains its DNA-binding and dimerization domains (the "TransActivation domain Mutant", TAM-67). We have shown that the TAM67 inhibits AP-1 activity and suppresses breast cancer growth [14,15]. We hypothesize that inhibition of AP-1 blocks the cell cycle, suppression of AP-1 in vivo inhibits the development of breast tumors, the cJun-DN protein binds and inactivates important growth regulatory proteins present in breast cancer cells, these proteins include Jun and Fos family members as well as coactivators that bind cJun. In the present study, we first showed that TAM67 inhibited breast cancer cell growth both *in vitro* and *in vivo*. We then investigated the mechanism by which TAM67 inhibits cell growth. We first demonstrated that TAM67 in breast cancer cells caused growth inhibition by suppressing G_1 cyclins expression and reducing CDKs activity, thus inducing a cell cycle block. We also showed that the expression of Tam67 induced apoptosis in the absence of serum. We then demonstrated that TAM67 binds all Jun and Fos family members, and it inhibits breast cancer cell also by interacting with cFos and by preventing the recruitment of co-activators.

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BODY

Statement of Work

Specific Aim 1: Determine the mechanism(s) by which AP-1 blockade inhibits breast cancer cell growth

Time Line	Task
Months 1-12	1). Determine whether AP-1 blockade affects the cell cycle and cell cycle regulators in breast cancer cells; 2). Determine whether AP-1 blockade induces apoptotic cell death and alters the expression of critical apoptosis regulators.

Specific aim 2. Identification of the Jun dimerization partners that are critical for the growth of breast cancer.

Time Line	Task
Months 13-16	To identify cJun-DN (TAM67)-binding proteins.
Months 17-24	To determine which of the cJun-DN-binding proteins are critical for breast cancer cells growth.

New specific aim 3. Determine whether differential recruitment of coactivators accounts for the ability of the TAM67 to inhibit AP-1 transcriptional activation .

Time Line	Task
Months 25-31	To compare the ability of cJun and TAM67 to bind coactivators, such as Jab1, AIB1, CBP, P300, and CAPER in MCF7 cells by using immunoprecipitation-Western blotting.
Months 32-36	To determine whether TAM67 alters recruitment of these coactivators by performing ChIP assays

PROGRESS:

Specific Aim 1. Determine the mechanism by which AP-1 blockade inhibits breast cancer cell growth.
1 a: Determine whether AP-1 blockade affects the cell cycle and cell cycle regulators in breast cancer cells. We first determined the effect of AP-1 blockade, TAM67, on cell growth by performing cell proliferation assay, we demonstrated TAM67 inhibit breast cancer cell growth. We next investigated the effect of AP-1 blockade on DNA synthesis and the cell cycle using a ^{3}H-thymidine incorporation assay and flow cytometry. The results of the ^{3}H-thymidine uptake assay showed that TAM67 dramatically inhibited ^{3}H-thymidine uptake in MCF-7 cells. Flow cytometry also showed that expression of cells of TAM67 reduced the proportion of cells in S phase, and increased the proportion in the G_0/G_1 phase. Thus, in the presence of serum, the expression of TAM67 blocked the cell cycle by causing a G_1 arrest. **1 b: Determine whether AP-1 blockade induces apoptotic cell death.** We first performed TUNEL assay to measure apoptosis. We found the expression of TAM67 dramatically increased the cell apoptotic rate in serum free condition, but there is no significant difference in full serum

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condition. To further demonstrate the involvement of apoptosis in the inhibition of MCF-7 cell growth, we then performed western blotting to measure the cleavage of PARP, a hallmark of apoptosis. When the MCF-7 Tet-Off TAM 67 cells were cultured in serum-containing medium, we also observed no PARP cleavage neither in TAM67 induced or uninduced conditions. Under serum–free condition, we did not see obvious PARP cleavage when TAM67 was not expressed, however, when TAM67 was induced the PARP cleaved band was clearly observed. Our study showed that TAM67 induced apoptosis in serum-free condition.

1 c: We have extended these studies to determine whether AP-1 Blockade alters the expression and/or activity of cell cycle regulators. We first performed western-blotting to determine the effect of TAM67 on Rb phosphorylation, we found that TAM67 caused Rb hypophosphorylation. We next performed luciferase assays to determine whether AP-1 blockade inhibits E2F activity. The results from these experiments demonstrated that TAM67 decreased E2F activity, and thus blocked the cell cycle. Next, we performed western-blotting to determine the effects of Tam67 on the expression of cell cycle regulatory proteins. We discovered that TAM67 decreased cyclin Ds (D1, D2, D3), cyclin E, CDK4, and CDK6 expression, while increase CDK inhibitor p27 expression. Next, we used CDK2 and CDK4 kinase assays to determine whether TAM67 reduced CDK activity. We demonstrated that both CDK2 and CDK4 activity were reduced by TAM67 expression. Our study suggests that TAM67 inhibits breast cancer growth predominantly by inducing inhibitors of cyclin dependent kinases (such as p27), suppressing G1 cyclins expression and reducing CDKs activity, thus inducing a cell cycle block.

Specific Aim 2: Identification of the Jun dimerization partners that are critical for the growth of breast cancer.

2a. To identify the known cJun-DN (TAM67)-binding proteins
First, using Western-blotting assay, I detected the expression of individual AP-1 family members in MCF7 Tet off TAM67 cells. I found that all Jun and Fos proteins were expressed in these cells. I then identified if TAM67 binds these AP-1 family members by immunoprecipitation-Western blotting assay. First, immunoprecipitation was done with an anti-Flag antibody (TAM67 is flag-tagged), and the precipitated proteins was analyzed using western blotting techniques with antibodies specific for individual Jun and Fos family members. Next, immunoprecipitation was done with antibodies specific for individual Jun and Fos family members, and the precipitated proteins was analyzed using western blotting with anti-Flag antibody. The data demonstrates that TAM67 binds all Jun and Fos family members.

2b. To investigate which of the cJun-DN-binding proteins are critical for breast cancer cell growth by using more selective cJun-DN mutants experiments.
It is known that the Jun-leucine zipper domain can bind Jun, Fos, ATF/CREB, Mafs, and coactivators to form homo- and heterodimerizations. However, the Fos-leucine zipper domain cannot bind Fos and ATF/CREB family members. Therefore we made specific mutant constructs of the cJun-DN that bind only Jun proteins or only coactivators, This mutant was used to narrow down potential candidates that bind cJun-DN.
2b.1. Make new mutant constructs of the cJun-DN by mutating its dimerization domain
I made cJun-DN/Fos mutant by replacing the cJun dimerization domain of the cJun-DN with Fos dimerization domain. Because it has Fos dimerization domain, cJun-DN/Fos can only heterodimerize with Jun family members and coactivators and cannot homodimerize with Fos

and ATF/CREB family members. I have also constructed a mutant called cJun-DN/Squelcher by deleting the dimerization domain of cJun. Because this protein does not have dimerization domain, it cannot dimerize with AP-1 family members, it may still bind coactivators.

2b.2. Introduce the Tet-off system into MCF7 cell line and screen clones expressing cJun-DN/Fos and cJun-DN/Squelcher mutants.

cJun-DN/Fos and cJun-DN/Squelcher mutants were transfected to MCF7 cells under control of a Tet-off system, selected the Flag-tagged cJun-DN/Fos and cJun-DN/Squelcher inducible clones and screen them for protein expression using Western blotting. The MCF 7 Tet-off cJun-DN/Fos and MCF 7 Tet-off cJun-DN/squelcher cell lines has been established.

2b.3. Investigate the effect of expression of cJun-DN/Fos and cJun-DN/Squelcher on AP-1 activity and the growth in MCF7 cells.

I did luciferase assays to determine whether cJun-DN/Fos and cJun-DN/Squelcher inhibit the AP-1 activity. I found that cJun-DN/Fos inhibited AP-1 activity while cJun-DN/Squelcher did not. I then performed proliferation assays to investigate whether cJun-DN/Fos and cJun-DN/Squelcher inhibit breast cancer cell growth. The data showed that both cJun-DN/Fos and cJun-DN/Squelcher did not affect the growth of MCF 7 cells.

These results indicate that proteins that bind cJun-DN/Fos are not critical for breast cancer cell growth. While proteins that bind TAM67 but not bind cJun-DN/Fos are critical for the growth of breast cancer cells. Thus, we hypothesized that Fos and ATF/CREB family members may be required for breast cancer cell growth.

2c. We have extended these studies to investigate the role of cJun and cFos on MCF 7 cell growth.

Antisense experiments were performed in this study. First, antisense cJun or cFos cDNA was used to block cJun or cFos expression in MCF 7 cells. I also found that antisense cJun and cFos cDNA inhibited AP-1 activity in these cells. Then colony formation assay and single cell proliferation assay were used to measure the effect of antisense cJun and cFos cDNA on MCF 7 cell growth. I observe that antisense cfos inhibited cell proliferation and colony formation, while antisense cJun did not. These results demonstrate that AP-1 transcriptional activity is critical for breast cell growth, and specifically that cFos expression is required for breast cancer cell growth.

Specific aim 3. Determine whether differential recruitment of coactivators accounts for the ability of the TAM67 to inhibit AP-1 transcriptional activation .

3a. Compare the ability of cJun and TAM67 to bind coactivators, such as Jab1, AIB1, CBP, P300, and CAPER in MCF7 cells.

First, using Western-blotting assay, I detected that Jab-1, AIB1, CBP, P300 were expressed in both MCF 7 Tet-off cJun and MCF 7 Tet-off TAM67 cells under doxycycline present and absent conditions. The expression of AIB1 is reduced when cJun is over-expressed. I then compared the ability of cJun and TAM67 to bind these coactivators. Immunoprecipitation was done with antibodies specific for Jab-1, AIB1, CBP, and P300, and the precipitated proteins was analyzed using Western-blotting with an anti-Flag antibody (both cJun and TAM67 are flag-tagged). I found that cJun binds all of these coactivators, while TAM67 only binds AIB1. These data suggest that TAM67 appears to inhibit breast cancer cell growth by preventing recruitment of co-activators. I have not been able to detect CAPER expression in MCF 7 cells because I have not got the antibody yet. To confirm these results I also performed the second way of

immunoprecipitation-Western assay which is to precipitate proteins by anti-Flag antibody and to analyze the proteins with antibodies specific for these coactivators. I found that cJun binds both Jab-1, P300 and AIB1, while TAM67 binds only AIB1. This result is consistent with my previous result.

3b. To determine whether TAM67 alters recruitment of these coactivators.

I performed ChIP assays using primers specific for the collagenase(MMP1) promoters to determine whether TAM67 alters recruitment of coactivators such as Jab-1, CBP, and P300. MCF 7 clones that stable express either cJun or TAM67 (Tet off cJun and MCF 7 Tet off TAM67 cell lines) under control of a Tet-off system were used in this experiment.

MCF 7 Tet-Off cJun and MCF 7 Tet off TAM67 cells were cultured in the presence or absence of doxycycline to induce cJun or TAM67 expression. First, western-blotting was performed to detect the expression of cJun, TAM67 in these cells. Then, ChIP Assay was performed to determine whether TAM67 alters recruitment of these coactivators compare to cJun. Cells were fixed, lysed after cross-link, the cell lysate was chopped to small pieces and incubated with antibodies specific for individual coactivator, the antibody-bound protein complex was precipitated using protein G sepharose beads, and The precipitated complex was heated to reverse the cross-link and followed by DNA purification. Finaly, PCR was performed using primers specific for the collagenase (MMP1) promoters.

I observed that Jab-1, CBP and P300 present in MMP1 promoter in MCF 7 cells when cJun is over-expressed, while none of them shows up when TAM67 is over-expressed. These results indicated that TAM67 prevents the recruitment of Jab-1, CBP and P300 in MCf 7 breast cancer cells.

KEY RESEARCH ACCOMPLISHMENTS

We studied the mechanism by which AP-1 blockade inhibits breast cancer cell growth. Our data demonstrated that the AP-1 blockade, TAM67, inhibited breast cancer growth both *in vitro* and *in vivo*. TAM67 suppressed cyclin D and E expression, increased p27 expression, decreased CDk2 and CDk4 kinase activity, caused Rb hypophosphorylation and reduced E2F activity, thus results a G1 cell cycle block leading to cell growth inhibition. We also observed that TAM67 induced MCF7 breast cancer cell apoptosis in serum free condition. Our recent data suggests that Jun and Fos family members are expressed in MCF 7 cells at TAM67 over-expressed condition, TAM67 binds all of these Jun and Fos family members. Our studies also showed that Jun families is not critical while cFos is required for breast cancer cell growth. TAM67 prevents the recruitment of co-activators. Therefore, TAM67 appears to inhibit breast cancer cell growth by interacting with cFos and preventing recruitment of co-activators.

REPORTABLE OUTCOMES

Specific aim 1:

1. The paper untitled "Inhibition of AP-1 transcription factor causes blockade of multiple signal transduction pathways and inhibits breast cancer growth" was published in Oncogene 21, 2002, Pages 7680-7689.
2. The manuscript "AP-1 Blockade in Breast Cancer Cells Causes Cell Cycle Arrest by Suppressing G1 Cyclin Expression and reducing cyclin dependent kinase activity" was published in Oncogene 2004, Sep 20.
3. Poster untitled "AP-1 inhibitor Causes a Cell Cycle Block by Inducing the Expression of CDK Inhibitors and by Suppressing the Expression of Cyclins D and E" was presented at *Era of Hope DOD Breast Cancer Research meeting, 2002.*
4. First year annual report had been submitted to funding agency.

Specific aim 2:

1. New MCF 7 Tet-Off inducible cell lines include MCF 7 Tet-Off TAM/Fos and MCF 7 Tet-Off TAM/Squelcher were established.
2. The manuscript "cFos is critical for breast cancer cell growth" is being submitted to ONCOGENE.
3. Second year annual report is submitted to funding agency.

Specific aim 3:

1. Poster untitled "AP-1 Blockade Inhibits Breast Cancer Cell Growth by Preventing the Recruitment of Coactivators" was presentated at AACR annual meeting 2003.
2. The manuscript "TAM67 prevents the recruitment of co-activators" is under preparation.
3. Third year annual report will be submitted to funding agency.

CONCLUSIONS

The cJun-dominant negative mutant, TAM67, inhibits breast cancer growth both *in vitro* and *in vivo*. Studies supported by this grant have shown that TAM67 inhibits breast cancer growth predominantly by inducing the expression of inhibitors of cyclin dependent kinases (such as p27), suppressing G1 cyclins expression and reducing CDKs activity, thus causing a cell cycle block. TAM67 also induces apoptosis in cells grown in serum free condition. We have also demonstrated that TAM67 binds all Jun and Fos family members, and that cFos is critical for growth of breast cancer cells. TAM67 inhibits breast cancer cell growth by by interacting with cFos and preventing recruitment of coactivators.

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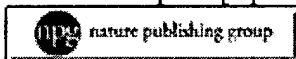
AP-1 blockade in breast cancer cells causes cell cycle arrest by suppressing G1 cyclin expression and reducing cyclin-dependent kinase activity.

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The AP-1 transcription factor is a central component of signal transduction pathways in many cells, although the exact role of AP-1 in controlling cell growth and malignant transformation is unknown. We have previously shown that AP-1 complexes are activated by peptide and steroid growth factors in both normal and malignant breast cells, and that blocking AP-1 by overexpressing a dominant-negative form of cJun (cJun-DN, TAM67) inhibits breast cancer cell growth both in vivo and in vitro. We hypothesized that TAM67 inhibits cell growth by altering the expression of cell cycle regulatory proteins, thus causing a cell cycle block. In the present study, we used clones of MCF7 breast cancer cells that express TAM67 under the control of an inducible promoter. First, we determined the effect of AP-1 blockade on cell growth, then we performed (3)H-thymidine incorporation and flow cytometry assays to investigate whether TAM67 inhibits the cell cycle. We observed that in the presence of serum TAM67 inhibited cell growth and caused a block in the G1 phase of the cell cycle. Next, we performed Western-blotting and CDK kinase assays to determine the effects of TAM67 on retinoblastoma (Rb) phosphorylation, the expression of cell cycle regulatory proteins, and CDK activity. We discovered that TAM67 inhibited Rb phosphorylation and reduced E2F activity. We also found that TAM67 decreased the expression of D and E cyclins, reduced CDK2 and CDK4 activity, and increased the CDK inhibitor p27. The studies of gene expression at the RNA level showed that TAM67 decreased cyclin Ds mRNA expression. Our study suggests that in the presence of serum, TAM67 inhibits breast cancer growth predominantly by inducing inhibitors of cyclin-dependent kinases (such as p27) and by reducing the expression of the cyclins involved in transitioning from G1 into S phase of the cell cycle. These studies lay the foundation for future attempt to develop new agents for the treatment and prevention of breast cancer.Oncogene advance online publication, 20 September 2004; doi:10.1038/sj.onc.1207889
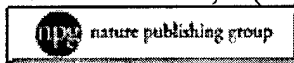
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Inhibition of AP-1 transcription factor causes blockade of multiple signal transduction pathways and inhibits breast cancer growth.

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AP-1 transcription factors play a critical role in signal transduction pathways in many cells. We have investigated the role of AP-1 in controlling proliferative signals in breast cells, and have previously shown that AP-1 complexes are activated by peptide and steroid growth factors in both normal and malignant breast cells. In this study, we investigated the role of AP-1 in transducing proliferative signals induced by peptide and steroid growth factors. We used MCF-7 clones that express a specific inhibitor of AP-1, a dominant-negative cJun mutant (TAM67), under the control of an inducible promoter to investigate the role of AP-1 in regulating breast cancer growth. In the presence of doxycycline (Dox), the AP-1 inhibitor was not expressed, and the MCF-7 clones proliferated normally in response to serum stimulation. However, when Dox was withdrawn, TAM67 was expressed, AP-1 activity was inhibited, and serum-induced proliferation was blocked. We next investigated whether the mitogenic response to specific growth factors also requires AP-1. MCF-7 Tet-Off-TAM67 cells were grown in the presence of increasing concentrations of IGF-1, EGF, heregulin-beta, bFGF, or estrogen under un-induced and induced conditions. These studies showed that the AP-1 inhibitor completely blocked proliferation in response to the peptide growth factors (IGF-1, EGF, heregulin-beta, and bFGF), and partially blocked the response to estrogen. To investigate the effect of AP-1 blockade on in vivo tumor growth, we injected the MCF-7 Tet-Off TAM67 cells into nude mice receiving doxycycline to suppress the expression of the AP-1 inhibitor. After the mice developed tumors, they were randomized to either continue to receive Dox or not. In mice not receiving Dox, the expression of TAM67 was induced, and tumor growth was inhibited, while the tumors in mice receiving Dox continued to grow. Analysis of the tumors from these mice showed that the expression of TAM67 caused reduced proliferation of the breast cancer cells without inducing apoptosis. These results demonstrate that AP-1 blockade supresses mitogenic signals from multiple different peptide growth factors as well as estrogen, and inhibits the growth of MCF-7 breast cancer cells both in vitro and in vivo. These results suggest that novel agents specifically targeting AP-1 or its activating kinases could be promising agents for the treatment of breast cancer.

cFos is Critical for MCF 7 Breast Cancer Cell Growth

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Running title : cFos is critical for the growth of breast cancer

Key Words: cFos, cJun, AP-1, breast cancer, antisense cDNA, cell proliferation

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Abbreviations: AP-1: Activating Protein-1; TAM67, cJun Dominant-negative mutant; DOX: Doxycycline; HRG-β, Heregulin-beta;

Abstract:

The AP-1 transcription factor is a central component of signal transduction pathways in many cells. We have previously demonstrated that blocking AP-1 by over-expressing a dominant negative form of cJun (cJun-DN, TAM67) inhibits breast cancer cell growth. We hypothesize that TAM67 binds and inactivates important growth regulatory proteins present in breast cancer cells. In the present study, we first demonstrated that TAM67 binds all of Jun and Fos proteins in breast cancer cells using immunoprecipitation-Western blotting. Next, we used 2 variants of this cJun mutant to investigate whether Jun or Fos members are required for breast cancer growth: TAM/Fos, in which the cJun dimerization domain was replaced by Fos dimerization domain, and TAM/Squelcher, in which the cJun dimerization domain was deleted. We isolated MCF7 cell lines that stably express these cJun-DN mutants under the control of an inducible promoter. By performing AP-1-dependent reporter assays, we observed that TAM67 and TAM/Fos inhibited AP-1 transcriptional activity, while TAM/Squelcher did not. We then determined whether TAM/Fos or TAM/Squelcher inhibits breast cell growth as does TAM67. We measured cell proliferation under induced and uninduced conditions and found that while TAM67 reppressed cell growth neither TAM/Fos nor TAM/Squelcher slowed cell growth. These results suggest that TAM67 (but not TAM/Fos or TAM/Squelcher) interacts with and inactivates proteins (such as Fos or ATF proteins), that are critical for cell growth. Finally, we performed antisense experiments to investigate whether cJun or cFos are required for breast cancer cell growth. Using antisense cDNA to block cJun or cFos expression and AP-1 activity, we assessed breast cancer cell growth after suppression of either cJun or cFos. We found that antisense cfos inhibited cell proliferation and colony formation, while antisense cJun did not.

These results demonstrate that AP-1 transcriptional activity is critical for breast cell growth, and specifically that cFos expression is required for breast cancer cell growth. The present results also provide the foundation for future studies to develop selective AP-1 inhibitors for the treatment or prevention of breast cancer.

Introduction

The AP-1 (activating protein-1) transcription factor is a key component of many signal transduction pathways. Although AP-1 was identified almost 15 years ago, the biological relevance and physiological functions of AP-1 and its components are still being elucidated. The AP-1 transcription factor is activated by peptide growth factors, hormones and stress signals (Chen *et al.*, 1996; Lin *et al.*, 2000; Webb *et al.*, 1999). The AP-1 family of transcription factors consists of Jun (cJun, JunB, JunD), Fos (cFos, FosB, Fra1, Fra2) proteins, Jun dimerization partners (JDP1 and JDP2), and also ATF (activating transcription factor) or CREB (CRE–binding) protein families, Maf proteins, and the neural retina specific gene product Nrl (Angel & Karin, 1991; Aronheim *et al.*, 1997; Karin *et al.*, 1997; Piu *et al.*, 2001; Vogt & Bos, 1990). These proteins contain a leucine zipper and form either homodimers or heterodimers through this domain. The Jun family members can homodimerize or heterodimerize with the different Fos, ATF/CREB family members, as well as v-Maf and c-Maf proteins. The Fos members can only heterodimerize with the different Jun family members. Jun-Jun and Jun-Fos dimers preferentially bind to the phorbol 12-O-tetradecanoate-13-acetate (TPA)- responsive element (TRE), whereas Jun-ATF dimers and ATF homodimers prefer to bind to the cAMP-responsive element (CRE) (Hai & Curran, 1991).

The differential expression and activation of the different Jun and Fos family members allow this transcription factors to control a wide variety of cellular functions. AP-1 has been implicated in many different biological processes, including cell differentiation, proliferation and

apoptosis (Brown *et al.*, 1993; Brown *et al.*, 1994; Ham *et al.*, 1995; Holt *et al.*, 1986; Rodgers *et al.*, 1994; Szabo *et al.*, 1991). Extensive analyses of mice and cultured cells have shown that such functional diversity can be achieved through the formation of dimers with different composition and biological properties.

AP-1 proteins have also been shown to be important regulators of growth in breast cancer cells. AP-1 has been shown to be variably expressed in human breast tumors, and AP-1 is modulated by many critical growth factors and hormones such as EGF, IGFs, estrogen, and retinoids (Chen *et al.*, 1996; Lin *et al.*, 2000; Schule *et al.*, 1991; Webb *et al.*, 1999). Increased levels of cJun and phospho-cJun in breast cancer tissue are also associated with low ER expression and tamoxifen resistance (Schiff *et al.*, 2000; Smith *et al.*, 1999). cJun overexpression in MCF7 breast cancer cells produces a tumorigenic invasive and hormone-resistant phenotype (Smith *et al.*, 1999). Previous studies from our lab have shown that a specific AP-1 inhibitor, the dominant-negative cJun mutant, TAM67, blocks AP-1 activity and inhibits breast cancer cell growth *in vitro* and *in vivo* (Liu *et al.*, 2002; Brown *et al.*, 1994; Ludes-Meyers *et al.*, 2001). We also found that TAM67 causes cell cycle arrest by suppressing G1 cyclin expression and reducing cyclin-dependent kinase activity (Liu *et al.*, 2004).

In the present study, to address the role of the proto-oncogenes cfos and cjun in regulating on breast cell growth, we developed 3 MCF7 cell lines that express inducible AP-1 mutants (TAM67, TAM/Fos, and TAM/Squelcher) under the control of the tet-off system. TAM67 is a cJun-dominant negative mutant (cJun-DN), that can dimerize with Jun, Fos, and ATF/CREB families. TAM/Fos is a Tam67 mutant in which cJun dimerization domain is replaced by Fos dimerization domain, thus it can only heterodimerize with Jun family members. It cannot homodimerize with Fos and ATF/CREB proteins. TAM/Squelcher is a TAM67 mutant

with no dimerization domain. Thus, it can not dimerize with any AP-1 family members. We report here the effect of the cJun mutants on breast cancer growth. We observed that TAM67 inhibited breast cancer cell growth, while TAM/Fos and TAM/Squelcher did not. We also determined the role of cJun and cFos in regulating breast cancer cell growth by performing antisense experiments. We found that cFos is critical for breast cancer cell growth. These studies contribute to our understanding of mechanisms of AP-1 action on breast cancer, and also provide the foundation for future studies to develop selective AP-1 inhibitors for the treatment or prevention of breast cancer.

Materials and Methods

Plasmids

The *TAM67*, *TAM/Fos*, and *squelcher* genes were constructed using the polymerase chain reaction (PCR) as described previously (Alani *et al.*, 1991; Brown *et al.*, 1994; Brown *et al.*, 1996). The correct sequence of each of these fusion genes was confirmed by sequencing using an automated DNA sequencer. All of these c-jun mutants were cloned into pUHD 10-3 5' Flag vector for further use.

Cell Culture, transfection, and cell lines established

The generation of the MCF7 Tet-off TAM67 clones has been previously described (Ludes-Meyers *et al.*, 2001). The cells were maintained in Improved MEM (high zinc option, Life Technologies, Grand Island, New York) with 100 ug/ml of geniticin and 100 ug/ml

hygromycin in presence of Doxicycline. The MCF7 Tet-off parental cells were maintained in Improved MEM (high zinc option, Life Technologies, Grand Island, New York) with 100 ug/ml of geniticin. The cells were transfected with Tam/Fos or Tam/Squelcher or vector alone using Fugene 6 reagent (Roche, Indianapolis, Indiana) according to manufacturer's recommendations, and stable clones were isolated after selection in hygromycin.

Luciferase Assay to Measure AP-1 activity

AP-1 transcriptional activity in cells was measured using the Dual-Luciferase[TM] Reporter Assay (Promega, Madison, Wisconsin) according to manufacturer's protocol. The cells were co-transfected with the Col-Z-Luc reporter gene containing the luciferase gene linked to 1100 bp of the human collagenase gene promoter which contains a single AP-1 binding site (TGAG/CTCA) and pRL-TK, a Renilla construct for normalizing of transfection efficiency. To determine the AP-1 activity stimulated by heregulin-beta (HRG-β), the cells were treated with HRG- β1 (10ng/ml, R&D System, Minneapolis, MO, USA) or DMSO respectively for 6 hours before harvest. Transfected cells were lysed 36 hours after transfection and luciferase activity was measured with equal amounts of cell extract using a microplate luminometer (Labsystems, Helsinki, Finland) and normalized with the Renilla activity.

Colony formation assay

This assay was performed as previously described (Ludes-Meyers et al., 2001). 2×10^5 cells were co-transfected in 6-well plate with 2ug of either pcDNA3.1 empty vector or pcDNA3.1 antisense cjun, or pcDNA3.1 antisense cfos. 12 hours after transfection the cells were split into 100mm dishes. 24 hours after transfection, G418 (Invitrogen) was added to a

final concentration of 800 ug/ml. After 2 weeks of selection in G418, resistant colonies were stained with crystal violet and counted. All experiments were performed in triplicate and the mean number of G418–resistant colonies were calculated.

Single cell proliferation assay

This assay was performed as previously described by Ludes-Meyers (Ludes-Meyers *et al.*, 2001). Cells were co-transfected with 0.2ug of pCMV-β-Gal and 2 ug of either pcDNA3.1 vector or pcDNA 3.1 TAM67, pcDNA3.1 antisense cjun cDNA, or pcDNA3.1 antisense cfos cDNA. 12 hours after transfection the cells were trypsinized to make a single cell suspension, and split into 100mm dishes. After 3 days, colonies of cells were fixed and stained with x-Gal to detect cells expressing β-Galactosidase *in situ*. Colonies containing blue cells were visualized by light microscopy and scored for the number of blue cells per colony. The number of blue cells per colony is a function of the growth rate of the transfected cells. The results were then plotted as the % colonies that showed 1 to 16 cells. Transfected genes that cause decreased growth reduce the number of blue cells within the colonies.

Cell proliferation assay of stably transfected Tet-off cell lines

The CellTiter 96^{TM} AQueous Non-Radioactive Cell Proliferation Assay (MTS assay; Promega, Madison, WI) was used to measure breast cancer cell growth according to the protocol provided by the manufacturer. Approximately 1000 cells were seeded in a 96 well plate. A solution containing a 20:1 ratio of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazo-lium) and PMS (phenazine methosulfate) was added to the cells for 2 hours at 37°C and absorption at 550 nm was determined. Each data point

was performed in quadruplet, and the results were reported as mean absorption +/- standard error.

Western blot assay

Cells were transfected with *cjun* or *cfos* antisense cDNA, or vector alone, 36 hours later, whole cell protein was extracted. Then the proteins were electrophoresed on a 10% acrylamide denaturing gel and transferred by electroblotting onto a nitrocellulose membrane. The cJun or cFos expression was detected by using following antibodies: Rabbit Polyclonal antibody specific for cJun (cat#: PC06, Oncogene Science, Cambridge, MA, 1:200); Mouse monoclonal antibody specific for cFos (cat#: sc-7202, Santa Cruz, 1:200).

Statistical analyses

The results of colony formation assays were expressed as mean number of colonies +/- standard error. Statistical significance was determined using the students's t-test. Single cell proliferation assays results in a distribution of cells per colony for each cell type. For this assay, Wilcoxon rank sum tests were used to compare distributions between antisense cjun- and vector-transfected cells or antisense cfos- and vector-transfected cells.

Results:

Construction of cJun mutants

To develop a panel of *cjun* mutants, we mutated each of the functional domains of cjun to produce the mutants shown in Fig.1. These mutants include TAM67 (Jun Δ3-122), TAM/Fos, and TAM/Squelcher. TAM67 is a cJun-dominant negative mutant (cJun-DN) that can dimerize with Jun, Fos, and ATF/CREB families. TAM/Fos is a Tam67 mutant in which the cJun dimerization domain is replaced by the Fos dimerization domain; thus it can only heterodimerize with Jun family members. TAM/Fos cannot homodimerize with Fos and ATF/CREB proteins. TAM/Squelcher is a TAM67 mutant with no dimerization domain. Thus, it can not dimerize with any AP-1 family members.

Expression of cJun mutants in MCF 7 cells

4 MCF7 cell lines (MCF7 Tet-off TAM67, MCF7 Tet-off TAM/Fos, MCF7 Tet-off TAM/Squelcher and MCF7 Tet-off vector cells) were generated as described in materials and methods. DOX was removed to induce the expression of cJun mutants, and protein lysates were prepared after 3 to 7 days after DOX removal. The proteins were then detected using western blotting. As shown in Figure 2, the cJun mutants were induced 3 to 7 days after DOX was removed.

Effect of cJun mutants on AP-1 activity

The cJun-dominant-negative mutant, Tam67, has previously been shown to inhibit AP-1 activity in several different cell lines (Liu *et al.*, 2004; Brown *et al.*, 1994; Ludes-Meyers *et al.*, 2001). In this study, we investigated whether the other cJun mutants,

TAM/Fos and TAM/Squelcher, affect AP-1 activity. The MCF-7 Tet-Off TAM/Fos (clones #28, #73), TAM/Squelcher (clones #25, #44), and vector transfected cells (clone #1) were cultured in the presence or absence of DOX to inhibit or stimulate the expression of TAM/Fos or TAM/Squelcher. As shown in Figure 3, TAM67 and TAM/Fos repressed the basal level of AP-1 activity (Fig 3a), and also inhibited AP-1 activity induced by heregulin (Fig. 3b) and TPA (data not shown). TAM/Squelcher did not affect basal or induced AP-1 activity (Fig.3).

Effect of cJun mutants on breast cancer cell growth

We have previously shown that TAM67 inhibits breast cancer cell growth (Ludes-Meyers *et al.*, 2001; Liu *et al.*, 2002). In the present study, we investigated whether TAM/Fos or TAM/Squelcher inhibits breast cancer cell growth. The MCF-7 Tet-Off TAM/Fos (clones #28, #24), TAM/Squelcher (clones #25, #44), and vector cells (clone #1) were cultured in the presence or absence of DOX for 7 days to inhibit or stimulate the expression of TAM/Fos or TAM/Squelcher, and cell proliferation was measured using the CellTiter 96TM Aqueous Non-Radioactive Cell Proliferation Assay (Promega Corporation, Madison, WI). Relative cell growth was then assessed by measuring absorbance at 620 λ wavelength using a spectrophotometric plate reader. As shown in Figure 4, neither TAM/Fos nor TAM/Squelcher inhibited breast cancer cell growth. These results suggest that TAM67 blocks critical pathways that TAM/Fos and TAM/Squlcher do not. One major difference between TAM67 and TAM/Fos is that TAM/Fos does not interact with Fos or ATF proteins. Thus, TAM67's ability to suppress cell growth depends on interacting with Fos or ATF proteins.

Effect of antisense cjun and antisense cfos on AP-1 activity

To determine whether cJun or cFos are required for breast cancer growth, we used antisense cDNA to inhibit cJun and cFos expression. MCF 7 cells were transfected with antisense cjun, or antisense cfos, or pcDNA 3.1 vector. As shown in Figure 5, cJun expression was blocked by antisense cjun cDNA, and cFos expression was blocked by antisense cfos cDNA. We next performed luciferase reporter assays using an AP-1-dependent reporter construct to determine the effect of antisense cjun and antisense cfos on AP-1 activity. As shown in Figure 6, we observed that both antisense cjun and antisense cfos reduced AP-1 activity.

Effect of antisense c-jun and antisense c-fos on breast cancer cell growth

We next investigated the role of *cjun* and *cfos* on breast cancer cell growth by performing colony formation assays and single cell proliferation assays in the presence of antisense *cjun* or *cfos* cDNA. In the colony formation experiments, our data demonstrated that antisense *cfos* significantly reduced colony formation ($p<0.01$), while antisense *cjun* caused a slight reduction in colony formation but was not significantly different than vector, as shown in Figure 7a. We observed similar results using the single cell proliferation assay (technique shown in Figure 7b). Antisense *cfos* significantly reduced single cell proliferation, while antisense *cjun* did not (Fig. 7c). These results show that cFos expression is critical for breast cancer growth. The results also suggest that while cJun may be important in regulating cell proliferation under some conditions, it is not absolutely required for *in vitro* growth of MCF 7 breast cancer cells. These antisense experiments are

also consistent with the growth studies of the inducible TAM67, TAM/Fos and TAM/Squelcher experiments described in Figure 4.

Discussion:

These studies were undertaken to investigate the relative importance of Jun and Fos proteins in regulating breast cancer cell growth. Our results demonstrate that a cJun dominant negative mutant, TAM67, inhibits breast cancer cell growth, while TAM/Fos and TAM/Squelcher do not. The major difference between TAM67 and TAM/Fos is that TAM67 dimerizes with Jun, Fos and ATF proteins, while TAM/Fos only dimerizes with Jun proteins. Thus, these results suggest that inactivation of Fos or ATF proteins is necessary to inhibit breast cancer cell growth. The antisense experiments further show that inhibition of cFos expression suppresses breast cancer cell growth, while inhibition of cJun expression does not. These results show that cFos is critical for breast cancer cell growth.

As a member of the AP-1 transcription factor complex, the cFos protein has been implicated as a key molecule in cell proliferation, differentiation, and transformation (Preston *et al.*, 1996). cFos protein can function as either a transcriptional activator or a transcriptional repressor depending on the cell type and condition. Modulation between the two functions has been postulated to be regulated through posttranslational modifications of the C-terminal region of the c-Fos protein, possibly through phosphorylation of serine residues (Barber & Verma, 1987). Previous experiments blocking cFos expression using antisense RNA or fos-specific antibodies resulted inhibition of cell proliferation in fibroblasts (Holt *et al.*, 1986; Nishikura & Murray, 1987; Riabowol *et al.*, 1988). The knockout of mouse *c-fos* gene demonstrated that c-fos is essential for bone formation, gametogenesis and certain neuronal functions. Homozygous c-fos-/- mice, although viable, are growth-retarded and develop osteopetrosis with deficiencies in bone

remodeling and tooth eruption (Johnson *et al.*, 1992). Results from other studies using microinjection of specific anti-Fos antibodies suggested that none of the Fos proteins individually is essential for cell proliferation in fibroblasts, but the neutralization of all four Fos member proteins blocked cell proliferation (Piechaczyk & Blanchard, 1994). In breast cancer studies, it has been showed that increased Fos protein expression has potential significance as a poor prognosis marker (Bland *et al.*, 1995), and XM6:anti*fos* antisense strategies appear beneficial in prolonging subject survival and inhibiting the proliferation and invasiveness of breast cancer xenograft systems(Arteaga & Holt, 1996; Robinson-Benion *et al.*, 1994). Furthermore, Gee *et al* have observed that significant associations between elevated Fos protein expression and increased proliferation, *de novo* endocrine insensitivity and a worsened prognosis in clinical breast cancer (Gee *et al.*, 1995). In addition to its primary role in normal development and cellular growth, the cFos protein has been associated with apoptotic cell death induced by antiproliferative conditions, and in response to cellular injury (Preston *et al.*, 1996). However, studies in *c-fos* knock out mice studies demonstrated that cFos is not essential for apoptosis (Gajate *et al.*, 1996; Roffler-Tarlov *et al.*, 1996). Our studies here are in agreement with the studies in fibroblasts- cFos is also critical for growth of breast cancer cells.

We have previously shown that a cjun dominant negative mutant, TAM67, can block AP-1 activity and inhibit breast cancer cell growth *in vitro* and *in vivo* (Liu *et al.*, 2002; Brown *et al.*, 1994; Ludes-Meyers *et al.*, 2001). TAM67 inhibits AP-1 activity by binding to and inactivating endogenous Jun or Fos proteins (Brown *et al.*, 1994; Brown *et al.*, 1996). We also previously shown that TAM67 causes cell cycle arrest by suppressing G1 cyclin expression, increasing P27 expression, and reducing cyclin-dependent kinase

activity (Liu *et al.*, 2004). Similar results have also been seen in human fibrosarcoma cells (Hennigan & Stambrook, 2001). The results show here extend these studies of TAM67 to show that a critical mechanism by which TAM67 suppresses growth is the inactivation of cFos.

Based on our results, we propose that suppression of AP-1 activity and specifically inactivation of cFos can inhibit breast cancer growth by modulating AP-1 dependent gene expression. We are currently attempting to identify the critical growth regulatory genes that are affected by cFos inactivation. Such studies will lead to a better understanding the role of AP-1 regulating breast cell growth, and will lead to new targets for the treatment and prevention of breast cancer.

Legends

Fig. 1. Schematic map of cJun and its dominant-negative mutants. TAM67 is a cjun dominant-negative mutant in which c-Jun transactivation domain was deleted. The leucine zipper domain of TAM67 was replaced with c-Fos to produce TAM/Fos. The leucine zipper domain of TAM67 was deleted to produce TAM/Squelcher.

Fig. 2. Expression of cJun mutants in MCF7 Tet-off cells. The MCF 7 Tet-off TAM67, MCF 7 Tet-off TAM/Fos, MCF 7 Tet-off TAM/Squelcher cells were cultured in the medium without DOX for 0-7 days, the protein expression was measured by Western-blotting. Actin was used as a loading control.

Fig. 3. Effect of the cJun mutants on AP-1 activity. The MCF7 Tet-off vector, MCF 7 Tet-off TAM67, MCF 7 Tet-off TAM/Fos, MCF 7 Tet-off TAM/Squelcher cells were cultured in the medium with or without DOX for 7 days to suppress or induce the expression of cJun mutants. The basal (Fig.3A) and HRG-β (Heregulin-beta) (Fig.3B) induced AP-1

activity were measured by luciferase assays. * Indicates statistical significant difference (P<0.01).

Fig. 4. Effect of cJun mutants on MCF7 cell proliferation. The MCF7 Tet-off vector, MCF 7 Tet-off TAM67, MCF 7 Tet-off TAM/Fos, MCF 7 Tet-off TAM/Squelcher cells were cultured in the medium with or without DOX for 7 days to suppress or induce the expression of cJun mutants, then cell proliferation was measured in next 0-8 days.

Fig. 5. Expression of cJun and cFos after antisense cDNA treatment. The MCF 7 cells were transfected with pcDNA3.1 alone or pcNDA3.1 antisense *cjun* or antisense *cfos*. After 2 weeks of selection, the whole cell lysate of transfected cells was used to measure cJun and cFos protein expression by western-blotting. cJun and cFos expression was reduced by antisense cDNA treatment.

Fig. 6. Effect of antisense *cjun* and *cfos* cDNA on AP-1 activity. The MCF 7 cells were transfected with pcDNA3.1 alone or pcNDA3.1 antisense *cjun* or antisense *cfos*. After 48 hours, the cells were lysate and AP-1 activity was measured by luciferase assays. Both antisense cjun and cfos cDNA reduced AP-1 activity. * Indicates statistical significant difference (P<0.005) between the effect of vector and antisense cjun or cfos.

Fig. 7. Cell proliferation assay of MCF7 breast cancer cells. A. Antisense *cfos* cDNA reduces colony formation in MCF 7 cells. MCF7 cells were transfected with pcDNA3.1 alone or pcNDA3.1 antisense *cjun* or antisense *cfos*. After 2 weeks of selection, survive colonies were stained with crystal violet and counted. The statistical significance of these results was analysed using 2-sample t-tests. B. Schematic diagram of the single cell proliferation assay. MCF 7 cells were co-transfected with pCMV-β-Gal and either pcDNA3.1 vector or pcDNA 3.1 TAM67, pcDNA3.1 antisense *cjun* cDNA, or pcDNA3.1

antisense *cfos* cDNA, after 3 days, the transfected cells were identified by staining in situ with X-gal and the number of transfected cells per colony were counted. C. Antisense *cfos* inhibits single cell proliferation in MCF 7 cells. MCF 7 cells were treated as described in B. The statistical significance of these results was analysed using the Wilcoxon rank sums test.

Acknowledgements

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